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**Preferential generation of 15-HETE-PE induced by IL-13
regulates goblet cell differentiation in human airway**

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Preferential generation of 15-HETE-PE induced by IL-13 regulates goblet cell differentiation in human airway epithelial cells

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Abstract

Rationale: Type-2-associated goblet cell hyperplasia and mucus hypersecretion are well known features of asthma. 15-Lipoxygenase (15LO1) is induced by the Type-2 cytokines/IL-13 in human airway epithelial cells (HAEC) *in vitro* and is increased in fresh asthmatic HAECs *ex vivo*. 15LO1 generates a variety of products, including 15-hydroxyeicosatetraenoic acid (15-HETE), 15-HETE-phosphatidylethanolamine (PE) (15-HETE-PE) and 13-hydroxyoctadecadienoic acid (13-HODE). The current study investigated the 15LO1 metabolite profile at baseline and after IL-13 treatment, and the influence on goblet cell differentiation in HAECs. **Methods:** Primary HAECs obtained from bronchial brushings of asthmatic and healthy subjects were cultured under air-liquid interface (ALI) culture supplemented with arachidonic acid (AA) and linoleic acid (LA) (10 μ M each) and exposed to IL-13 for 7 days. siRNA transfection and 15LO1 inhibition were applied to suppress 15LO1 expression and activity. **Results:** IL-13 stimulation induced 15LO1 expression and preferentially generated 15-HETE-PE *in vitro*, both of which persist after removal of IL-13. 15LO1 inhibition (siRNA and chemical inhibitor) decreased IL-13 induced FOXA3 expression, while enhancing FOXA2 expression. These changes were associated with reductions in both MUC5AC and periostin. Exogenous 15-HETE-PE stimulation (alone) recapitulated IL-13 induced FOXA3, MUC5AC and periostin expression. **Conclusions:** The results from this study confirm the central importance of 15LO1 and its primary product 15-HETE-PE to epithelial cell remodeling in HAECs.

Keywords: asthma, mucus hypersecretion, eicosanoid, 15-Lipoxygenase-1, phospholipid

47 **Introduction**

48

49 Goblet cell hyperplasia and associated mucus hypersecretion are well known features of asthma
50 which contribute to its morbidity and mortality. It is particularly seen in those patients with evidence
51 for Type-2 (IL-4/-13) associated inflammation (1) (2, 3). This Type-2 process is believed to
52 contribute to differentiation of basal epithelial cells into a goblet cell/mucus-producing epithelium.
53 Previous studies further showed that MUC5AC was the major mucin increased in human airway
54 epithelial cells (HAEC) in response to Type-2/IL-13 stimulation *in vitro* (4-10). Goblet cell
55 differentiation appears to be tightly regulated by forkhead box protein transcription factors,
56 including FOXA3 (for goblet cell differentiation) (11) and FOXA2 (associated with ciliated cell
57 differentiation). FOXA3 is strongly upregulated by IL-13 (11, 12). However, the pathways by which
58 IL-13 stimulates these downstream events are not known.

59 Type-2 immunity impacts additional epithelial factors, including 15-lipoxygenase. As the only
60 lipoxygenase class expressed by HAECs (13), 15-lipoxygenases (15LOX) catalyze oxygenation
61 of polyunsaturated fatty acids, inserting molecular oxygen at the C15 position of arachidonic acid
62 (AA) or the C13 position on linoleic acid (LA) to produce 15S-hydroxyeicosatetraenoic acid [15(S)-
63 HETE (AA)], or 13S-hydroxyoctadecadienoic acid [13(S)-HODE (LA)]. In humans, two distinct
64 subtypes of 15LOX exist: 15LO1 (ALOX15) and 15LO2 (ALOX15B) with different tissue and
65 cellular distribution (14-16) and possible differences in substrate preferences. While 15LO2
66 exclusively oxygenates AA to generate 15(S)-HETE, with poor catalytic activity on LA (17), human
67 reticulocyte 15LO1 has been reported to prefer LA in a cell-free system (18). 15LO1, and its
68 product, 15-HETE are known to be elevated in asthmatic lungs, and upregulated by IL-4/-13 *in*
69 *vitro* (10, 19, 20), while 15LO2 is not (14, 19, 21). Our studies also showed that IL-4/-13 induce

70 15LO1 expression associated with generation of 15-HETE conjugated with PE (15-HETE-PE) in
71 both monocyte/macrophages and epithelial cells (10, 22). This predisposition to 15-HETE-PE (as
72 opposed to free 15-HETE) generation is seen in the presence of interactions with PEBP1,
73 MAPK/ERK activation and MUC5AC expression (10, 23). However, the balance of the LA or AA
74 products, 13-HODE, 15-HETE or their esterified forms, including 15-HETE-PE, as well as their
75 contribution to cell differentiation and mucus hypersecretion in HAECs has not been evaluated.

76 Finally, IL-13 is known to upregulate additional factors which associate with remodeling in the
77 airway epithelium, including periostin which has been identified as a Type-2 biomarker in both
78 HAECs and serum (24) (25). It is associated with matrix deposition and is likely part of a wound-
79 repair process, similar to mucin generation.

80 We therefore hypothesized that 15LO1 would preferentially metabolize AA to generate
81 phospholipid conjugated 15-HETE-PE, as opposed to free 15-HETE (or 13-HODE) in response to
82 IL-13, which would regulate IL-13 induced goblet cell differentiation. To address this hypothesis,
83 cultured HAECs stimulated with IL-13 and supplemented with AA/LA were evaluated for free and
84 conjugated lipid products by LC/MS. The stability of 15LO1 expression and activity were evaluated
85 as well as the effects of 15LO1 and its product 15-HETE-PE, on goblet cell differentiation and
86 periostin expression (24) (25).

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91 **Materials and methods** (*Please see online supplement for more complete methods*)

92 **Reagents, antibodies and primers**

93 ALOX15 DsiRNA™ was purchased from IDT (Coralville, IA). Antibodies against FOXA3 (goat IgG)
94 and periostin (rabbit IgG1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and
95 anti-GAPDH antibody was from Novus Biologicals (Littleton, CO). Anti-MUC5AC antibody was
96 from Neomarkers (Fremont, CA). 15LO1 antibody was a gift from Dr. Doug Conrad, University of
97 California, San Diego(26). BLX2477, a highly specific inhibitor of 15LO1, was a kind gift from Dr.
98 Hans-Erik Claesson (27). All other antibodies and reagents used are described in Online
99 *Supplement Materials and Methods*.

100 **Sources of HAECs**

101 HAECs were obtained by bronchoscopic brushing of asthmatic and healthy control (HC) airways
102 as previously described (28). *See detail in Supplement Materials and Methods online*.

103 **Primary Human Airway Epithelial Cell (HAEC) Culture in Air–Liquid Interface, DsiRNA**
104 **Transfection and exogenous 15-HETE-PE stimulation**

105 HAECs were cultured in air–liquid interface (ALI) as previously described (23, 29), and DsiRNA
106 transfection was performed using Lipofectamine transfection reagent, with details in *Supplement*
107 *Materials and Methods online*. LA/AA supplementation and exogenous 15-HETE-PE stimulation
108 were performed as described in Online *Supplement Materials and Methods*.

109 **15LO1 enzymatic inhibition with BLX2477**

A selective enzymatic inhibitor of 15LO1 (BLX2477) was applied to IL-13 treated or untreated HAECs (27). BLX2477 (2 μ M) was added acutely for 1 hour or up to 5 days before harvest (27). For longer duration studies, the culture medium and BLX2477 were changed every 24 hours.

Liquid Chromatography/UV/Mass Spectrometry Analysis

Cells were kept in PBS buffer containing diethylenetriaminepenta-acetic acid (DTPA) (100 μ M) and butylated hydroxytoluene (BHT, 100 μ M). Free and esterified HETEs were analyzed by LC/MS as previously described (30, 31).

Western Blotting

Cell lysates were run on 4-12% SDS-PAGE gels under reducing conditions and protein detected as previously described (32).

Real-time PCR

Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems) using primers and probes from Applied Biosystems, with GUSB as the internal control. An identical threshold cycle (Ct) was applied for each gene of interest. Relative mRNA expression levels were calculated using the delta Ct method.

Semiquantitative MUC5AC ELISA

MUC5AC protein was measured from apical culture supernatants using a semiquantitative sandwich ELISA with two different MUC5AC antibodies. All results are in relative arbitrary units per ml (AU/ml), and were reported as fold change in relative to corresponding control condition. See additional details in Online Supplement Materials and Methods.

130 **Statistical Analysis**

131 Statistical analysis was performed using JMP SAS software (Cary, NC). Data that were normally
132 distributed were represented as means \pm SEM. Each “n” identifies the number of biologically
133 replicated experiments from different donors. For each donor condition, technical replicates were
134 generally run in triplicate, except for western blots, which were done in singlets due to limitation of
135 sample. Cells from asthmatic donors are identified by dashed lines, while HC donors are identified
136 by solid lines. All comparisons of cells from specific donors, under 2 different conditions, i.e
137 scramble and siALOX15, are run using paired T-test. P-values of <0.05 were considered
138 statistically significant.

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Results

Demographics of research participants providing HAECs for culture

Fresh human airway epithelial cells for ALI culture were obtained from a total of 44 subjects (*Table S1*). Due to the limited cell numbers and longer term development of the experimental models, donor sources for each experiment varied. However, as reported previously, studies of this pathway *in vitro* have not identified differences in response by subject group (asthma vs healthy control) (10, 22). Thus, cells from different subject groups were used interchangeably with subject group of donor cells identified on the figures: asthmatics and HCs shown by dashed and solid lines respectively. There were no differences in response by age.

15LO1 expression and specific enzymatic activity in HAECs

IL-13 stimulation induces 15LO1 expression and preferentially increases 15-HETE-PE in the presence of equal LA/AA supplementation in vitro

15LO1 has been reported to directly oxygenate endogenous PE to produce esterified 15-HETE-PE in response to IL-13 stimulation. To determine whether 15LO1 generates additional lipid products in HAECs under IL-13 conditions, we stimulated HAECs with IL-13 for 7 days. Intracellular (cell lysates) and extracellular (medium) levels of 15-HETE-PE, 13-HODE-PE, 13-HODE and 15-HETE were measured. To confirm that product specific generation was not dependent on intracellular depletion of LA and/or AA, culture media were supplemented with equal amounts (10 μ M) of exogenous LA and AA under both basal and IL-13 conditions. As expected, IL-13 induced high levels of intracellular 15-HETE-PE (25.7 ± 3.6 ng/million cells, 17.5 ± 1.4 fold change over baseline, $n=8$, $p<0.0001$), with only modest increases in 13-HODE or free 15-HETE in cells under unsupplemented basal culture conditions (Figure 1a). Exogenous LA/AA

173 supplementation under basal conditions only modestly increased the overall levels of 15-HETE-
174 PE (23.3 ± 4.1 ng/million cells), 15-HETE (7.4 ± 3.8 ng/million cells) and 13-HODE (8.9 ± 4.3 ng/million
175 cells) without alteration in their ratios to each other as compared to the unsupplemented basal
176 condition ratios. In contrast, exogenous LA/AA supplementation of IL-13 stimulated cells
177 generated marked increases in 15-HETE-PE (186.1 ± 25 ng/million cells, 14.5 ± 2.0 fold change over
178 baseline, $n=9$, $p<0.0001$), with only modest increases in intracellular 13-HODE (9.8 ± 2.3 ng/million
179 cells, 1.9 ± 0.3 fold change, $n=9$, $p<0.02$) and 15-HETE (8.7 ± 1.6 ng/million cells, 1.6 ± 0.2 fold
180 change, $n=6$, NS) (Figure 1b). There were no differences in response by subject group under any
181 conditions. Extracellular (media) 13-HODE and 15-HETE levels were both low at baseline and
182 increased modestly in response to IL-13 (*See supplemental Figure S1*). As previously reported,
183 no significant level of 15-HETE-PE was detected extracellularly with or without LA/AA
184 supplementation (10, 22). Additionally, 13-HODE-PE was undetectable either extracellularly or
185 intracellularly in any condition, with or without LA/AA supplementation.

186

187 To determine whether the increase in 15-HETE-PE following IL-13 was associated with increased
188 activity of 15LO1 or 15LO2, the levels of each protein were measured in IL-13 stimulated HAECs.
189 As shown in Figure 1c, IL-13 markedly upregulated 15LO1 without effect on 15LO2. LA/AA
190 supplementation did not impact 15LO1 and 15LO2 protein levels. 15LO1 protein was then
191 knocked down by DsiRNA transfection (siALOX15) and 15-HETE-PE measured. Knockdown of
192 15LO1 dramatically inhibited 15LO1 protein expression (Figure 1d, $n=4$) and 15-HETE-PE
193 generation ($n=11$, $p=0.001$) (Figure 1e).

194

195 To confirm the importance of 15LO1, the effect of a selective 15LO1 enzymatic inhibitor BLX2477
196 was also studied. Primary HAECs were dosed with BLX2477 ($2 \mu\text{M}$) or vehicle control (DMSO)

for 1 hour for acute studies or every 24 hours up to 5 days to observe chronic effects. BLX2477 decreased 15-HETE-PE generation as early as 1 hour after treatment (Figure 1f, n=8, p=0.023). Generation of 15-HETE-PE continued to be suppressed for up to 5 days of BLX2477 treatment (Figure 1g, n=3, p=0.041). Interestingly, 15LO1 protein (but not mRNA) was also suppressed by BLX2477 as compared to DMSO after 5 days treatment (Figure 1h, n=5, p=0.0002), suggesting a positive feedback mechanism through 15LO1 metabolites, but possibly also due to off-target effects of BLX2477 (27).

15LO1 protein levels and activity persist in the absence of IL-13

To determine the stability of IL-13 induced 15LO1 enzyme and activity, cells were stimulated with IL-13 for 7 days. IL-13 was then removed for the remaining culture period up to 72 hours. As shown in Figure 2a, IL-13 induced 15LO1 mRNA levels, which rapidly returned to basal levels after removal of IL-13. In contrast, high levels of 15LO1 protein remained for at least 72 hours following removal of IL-13 (Figure 2b). Additionally, intracellular 15-HETE-PE levels remained elevated over baseline after IL-13 removal (Figure 2c). These results suggest the sustained presence of an active enzyme in the absence of Type-2 stimulation for extended periods of time.

15LO1 pathway and Epithelial Remodeling

The 15LO1 pathway regulates IL-13 effects on FOXA3 and FOXA2

To determine whether 15LO1 expression and activity were upstream of the Forkhead-box proteins FOXA3 and FOXA2 and central to goblet cell differentiation, DsiRNA 15LO1 knockdown was performed and FOXA3, FOXA2 and MUC5AC analyzed. Confirming previous work, FOXA3 protein expression increased in a time dependent manner in ALI culture (*supplemental FigureS2a*). IL-13 induced high levels of FOXA3 mRNA and protein, which paralleled the increase of 15LO1

(supplemental Figure S2a, b). In contrast, FOXA2 mRNA was suppressed by IL-13 stimulation (supplemental Figure S2c). 15LO1 knockdown significantly decreased FOXA3 mRNA and protein expression (Figure 3a, b). As a marker of goblet cell differentiation, IL-13 induced MUC5AC expression was also suppressed by 15LO1 knockdown (Figure 3c, d). In contrast, 15LO1 knockdown upregulated FOXA2 mRNA expression (Figure 3e). These results were confirmed utilizing the selective 15LO1 enzymatic inhibitor BLX2477 (Figure 4). Similar to the results with 15LO1 knockdown, BLX2477 treatment significantly inhibited IL-13 induced FOXA3 mRNA (Figure 4a) and protein (Figure 4b). IL-13 induced MUC5AC mRNA (Figure 4c) and MUC5AC protein secretion (Figure 4d) were also inhibited by BLX2477 treatment. Similar to 15LO1 knockdown, FOXA2 mRNA was upregulated by BLX2477 (Figure 4e).

231

15LO1 pathway inhibition decreases periostin expression and secretion

To determine whether 15LO1 expression and activity may be involved in other known Type-2 pathways in HAECs, the impact of 15LO1 pathway inhibition on periostin was investigated. As expected, IL-13 induced both periostin mRNA (Figure S3a) and protein expression (Figure S3b) as well as secretion (Figure S3c). 15LO1 DsiRNA knockdown inhibited IL-13 induced periostin mRNA expression (Figure 5a) as well as intracellular (Figure 5b) and secreted periostin protein (Figure S4a). Similarly, BLX2477 (2 μ M) treatment decreased periostin mRNA (Figure 5c) as well as intracellular (Figure 5d) and secreted protein (Figure S4b) after 5 days of exposure. Thus, the 15LO1 pathway regulates a range of downstream Type-2 pathways beyond those related to goblet cell differentiation.

242

Exogenous 15-HETE-PE stimulation induces FOXA3, MUC5AC and periostin expression in HAECs in the absence of IL-13

244

245 To determine whether products of an activated 15LO1 pathway, in the absence of IL-13, were
246 sufficient to induce goblet cell differentiation and periostin expression, 1 μ M 15-HETE-PE (or
247 DMPE control) was added to HAECs in ALI every 48 hours up to 5 days. As shown in Figure 6,
248 exogenous 15-HETE-PE induced FOXA3 (Figure 6a, b) as well as MUC5AC mRNA and protein
249 expression (Figure 6c, d). Exogenous 15-HETE-PE stimulation also induced periostin mRNA and
250 protein expression (Figure 6b, e). In contrast, there was no significant effect on FOXA2 (Figure
251 6f). These data directly support both a necessary and sufficient role for 15-HETE-PE in regulation
252 of goblet cell differentiation and periostin expression. However, under the specific conditions
253 studied, the effects of exogenous 15HETE-PE were substantially more modest as compared to
254 IL-13 stimulation.

255 **Discussion**

256 The results from this study confirm the critical importance of 15LO1 and its primary product 15-
 257 HETE-PE to epithelial cell remodeling associated with asthmatic airways. 15LO1 is the primary
 258 lipoxygenase induced in response to the Type-2 cytokine IL-13, and 15-HETE-PE is the primary
 259 product. Interestingly, this pathway remains present and active for extended periods of time
 260 despite removal of IL-13. Using knockdown, selective enzyme inhibitor and exogenous addition
 261 approaches, the results presented here confirm that the 15LO1 pathway regulates goblet cell
 262 differentiation, not only increasing expression of FOXA3, critical for goblet cell differentiation, but
 263 also preventing expression of pathways critical for ciliated cell formation (11, 33). Finally, 15LO1
 264 also impacts a broader range of Type-2/remodeling associated gene expression than just those
 265 associated with goblet cell differentiation, including a profound effect on periostin expression as
 266 well.

267
 268 Lipoxygenase enzymes can act on a variety of fatty acid substrates, commonly including AA and
 269 LA, to produce a range of oxygenated lipids. Human 15LO1, the most abundant lipoxygenase
 270 present in HAECs under TH2 conditions, has been reported to prefer LA over AA to generate 13-
 271 HODEs using the competitive substrate capture method in a cell-free system (18). In HAECs,
 272 however, IL4/-13 preferentially increases (14, 19, 21) a phospholipid (PE) conjugated form,
 273 15HETE-PE, in both monocyte/macrophages and epithelial cells (10, 22). Our previous studies
 274 confirmed that IL-13 stimulated 15LO1 interacted with PEBP1 to further induce MAPK/ERK
 275 activation and MUC5AC expression (10, 23). This suggests that different lipid products of 15LO1
 276 pathway generated by specific substrate may play different biological roles in HAECs. However,
 277 it is unclear whether LA products, such as 13-HODE could also be playing a role. In the present

study, IL-13 stimulated HAECs supplemented with equal amounts of AA and LA, were evaluated for free and conjugated lipid products profiles by LC/MS.

Lipoxygenases traditionally oxygenate free fatty acids, arachidonic acid originating predominantly from arachidonoyl-phospholipids following activation of phospholipases (34, 35). In the presence of free arachidonic acid (AA), this leads to the insertion of molecular oxygen at the C15 position and production of the unstable 15-hydroperoxy-eicosatetraenoic acid (15-HpETE), which is rapidly reduced to stable 15-HETE by one of glutathione peroxidases (eg, glutathione peroxidase 4 (GPX4) (36, 37). However, unlike other lipoxygenases, 15LO1, under certain conditions, appears to alter its substrate preference from free AA to arachidonic acid containing phospholipids, resulting in high levels of 15-HETEs conjugated to phospholipids, particularly 15-HETE-PE (10, 22, 30). In the present study, 15LO1 activity, under IL-13 conditions preferentially increases 15-HETE-PE, with only minimal increases in free 15-HETE or 13-HODE with no detectable 13-HODE-PE, despite previous studies suggesting LA is a preferred substrate in a cell-free system. This difference could be accounted for by the in vitro vs cell free systems, and the experimental conditions applied. Since our previous study showed that 15-HETE-PE regulates MAPK/ERK activation and MUC5AC expression in HAECs (10, 23), the disproportionate increase in 15-HETE-PE suggests it plays a role in the goblet cell hyperplasia and mucus hypersecretion associated with asthma. At the same time, the robust increase in 15-HETE-PE in the presence of free AA further suggests that the AA is being incorporated into membrane phospholipids prior to being acted on by IL-13. Alternatively, 15LO1 may oxygenate free or esterified AA to produce 15-HpETE that is subsequently reacylated into lyso-PE.

15 LO1 has also been identified as an enzyme critical for generation of hydroperoxy-phospholipids, when 15LO1 switches from metabolizing free PUFAs to esterified AA-phospholipids (38). Generation of these intracellular esterified AA-phospholipids, particularly 15-HpETE could lead to a newly identified cell death termed ferroptosis (10, 30) (38). Thus, in addition to controlling cell differentiation, this switch to generation of 15-HpETE-PE (and 15-HETE-PE) could also control cell survival and death. However, the mechanisms by which 15LO1 changes its preference from free to phospholipid conjugated fatty acids, in particular AA, requires further study.

Another important finding is the stability of 15LO1 protein and its enzyme activity. 15LO1 and its eicosanoid product 15-HETE have long been noted to be increased in human asthma in relation to severity and eosinophilic inflammation (14, 19, 39). 15LO1 is induced by Type-2 cytokines in monocytes/macrophages and in HAECs is one of the genes most strongly induced by IL-13 *in vitro* (20, 40, 41). Despite the very high expression of Type-2 signature genes like 15LO1, the levels of the presumed Type-2 cytokines, IL-4 and -13 (mRNA and protein) in asthmatics, especially those more severe patients treated with corticosteroids, are low (42, 43). The reasons for this disconnect between high levels of 15LO1 and low levels of Type-2 cytokines/IL-13 *in vivo* are not clear. However, the high, sustained 15LO1 protein levels and activity following removal of IL-13, as observed in this study, could contribute to prolonged IL-4/-13 downstream pathway activity, even in the absence of these cytokines. This long half-life appeared to be limited to protein, as mRNA levels fell rapidly. In contrast, high levels of 15LO1 protein remained for at least 72 hours following removal of IL-13. More importantly, intracellular 15-HETE-PE levels also remained elevated over baseline after IL-13 removal. Given that the media is changed every 2 days, this represents newly formed 15-HETE-PE. Thus, the enzyme is functional and contributing newly

324 formed lipid mediators long after removal of the IL-13, potentially explaining the high levels
325 measured *in vivo*.

326 We previously reported that 15LO1, conjugated with the scaffolding protein PEBP1, is critical for
327 IL-13 induced HAEC MUC5AC expression. In addition, 15LO1 activity modulated eotaxin-
328 3/CCL26 and inducible NO synthase (23). However, its broader effects on Type-2 cytokine
329 induced goblet cell differentiation/function are not known. Given its high levels and sustained
330 activity, we hypothesized it would have a broad and central role. Recent studies have suggested
331 that goblet cell differentiation is critically dependent on a specific forkhead DNA-binding protein
332 (FOXA3) which binds to proximal promoters of several groups of genes associated with goblet cell
333 metaplasia (11, 33) as well as suppression of FOXA2 (11, 12, 44). Both HAECs and transgenic
334 mice studies confirmed FOXA3 induced goblet cell metaplasia and enhanced expression of
335 MUC5AC. Similarly, a recent microarray study of fresh HAECs from healthy and asthmatic
336 subjects demonstrated high FOXA3 mRNA levels in Type-2 associated asthma, which correlated
337 strongly with MUC5AC (and 15LO1) (39). In contrast, FOXA2 has been reported to suppress
338 goblet cell metaplasia, while inducing ciliated cell differentiation (12). Interestingly, results
339 presented here clearly show that expression and activity of the 15LO1 pathway, using chemical
340 inhibitors, DsiRNA, and exogenous addition of 15-HETE-PE control IL-13 induced FOXA3. At the
341 same time, FOXA2 expression was inhibited, supporting an overall effect of 15LO1 to influence
342 remodeling of the airway epithelium in favor of goblet cells consistent with the microarray data (10,
343 39). The lack of effect of exogenous 15-HETE-PE on FOXA2 expression requires further study,
344 but may be related to both dose and uptake of the exogenous hydroxyl-phospholipid into the cells.
345 The mechanisms by which 15LO1 expression/activity control the expression of these transcription
346 factors remains speculative, but could include ERK/MAP kinase activation or interaction of

347 membrane phospholipids with the IL-4 receptor alpha or its downstream signaling pathways,
348 including MEK-ERK. Further study is needed.

349

350 To begin to determine the extent to which 15LO1 controlled prominent IL-13 induced pathways,
351 we also evaluated the impact of the 15LO1 pathway on HAEC periostin expression. Periostin is
352 an extracellular matrix protein which can regulate adhesion and migration of epithelial cells in
353 airway remodeling in asthma and other diseases (45, 46). Multiple studies report elevations in
354 periostin mRNA in freshly brushed HAECs and serum from patients with Type-2 high asthma (24,
355 25). Our results show that IL-13 induced high levels of periostin expression and secretion in
356 HAECs consistent with previous studies. More importantly, our results show a marked inhibition
357 of periostin expression in the face of 15LO1 inhibition which parallels the reductions in goblet cell
358 differentiation as indicated by MUC5AC expression, and are mirrored by increases in periostin
359 expression after exogenous 15-HETE-PE addition. These results suggest periostin expression is
360 associated with goblet cell differentiation and mucus hypersecretion in response to IL-13
361 stimulation in HAECs. Interestingly, in contrast to the findings reported here, periostin was recently
362 suggested to suppress goblet cell metaplasia in a mouse model, with periostin knock-out mice
363 demonstrating increased differentiation of epithelial cells into mucus-producing goblet cells upon
364 sensitization and challenge with OVA without effect on allergic inflammation (47). Thus, in mice,
365 an opposite effect for periostin on goblet cell metaplasia appears to be observed. Although the
366 reasons for the differences in HAECs and in the mouse model are unclear, species-specific
367 epithelial differences, the knockout models used, as well as the differences in the challenges could
368 be explanations (47).

369

While the studies presented here strongly support development and testing of 15LO1 pathway inhibitors in asthma, replication of these findings in transgenic mice would add to the functional importance of the pathway. However, given the large differences between mouse and human airway epithelial cells and even structure, substantial studies to confirm the importance of this pathway in mice as compared to human airways will be required, including confirmation of whether mouse airway epithelial cells even express 15LO1, or the mouse equivalent 12/15LO1. Previous studies in transgenic mice have not commented on epithelial expression despite suppression of mucus (48).

Summary and Conclusions

The data presented here confirm a critical role for 15LO1 and its product 15-HETE-PE in HAEC goblet cell differentiation. Under IL-13 conditions, 15LO1 is the most abundant 15-lipoxygenase present in cultured HAECs, where it remains at high levels and active for prolonged periods of time even in the absence of IL-13. This IL-13 induced 15LO1 strongly favors the metabolism of arachidonic acid, conjugated with phospholipids over free fatty acids to form 15-HETE-PE, the process of which appears to be both necessary and sufficient to impact FOXA3 and periostin expression and goblet cell differentiation *in vitro*. Further studies are needed to better understand the mechanisms by which this pathway controls these epithelial changes, as well as functional implications of this pathway to human diseases like asthma.

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Figure Legends

Figure 1. IL-13 stimulation induces 15LO1 expression and preferentially increases 15-HETE-PE in the presence of equal LA/AA supplement in vitro and is inhibited by DsiRNA transfection and chemical inhibition of 15LO1. HAECs were stimulated with IL-13 for 7 days with/without LA/AA supplements, and cell lysates collected for 15-HETE-PE, 13-HODE-PE, free 13-HODE and 15-HETE measurement by LC/MS and 15LO1/2 protein detection by Western-blot. *Solid lines: healthy controls; Dashed lines: asthmatics.* (a) IL-13 induced high levels of intracellular 15-HETE-PE, with only modest increases in 13-HODE and free 15-HETE under basal conditions without exogenous LA/AA supplementation. (b) supplementation with LA/AA further increased IL-13 induced generation of 15-HETE-PE, with only modest increases in 13-HODE and 15-HETE. No 13-HODE-PE was detected. (c) Representative western-blot and densitometry (n=3) show IL-13 induced 15LO1 expression without effect on 15LO2, and LA/AA supplementation did not impact 15LO1 and 15LO2 protein levels. (d) DsiRNA knockdown of 15LO1 inhibited 15LO1 protein expression (n =4) and (e) 15-HETE-PE generation. The selective 15LO1 enzymatic inhibitor BLX2477 suppressed 15-HETE-PE generation at 1 hour (f) and 5 days of treatment (g), and (h) BLX2477 suppressed 15LO1 protein expression at 5 days of treatment.

Figure 2. IL-13 induced 15LO1 and 15-HETE-PE remain stable after the removal of IL-13. HAECs were all stimulated with IL-13 for 7 days except the control condition. IL-13 was removed for the remaining culture period up to 72 hours. Cell lysates were collected for 15-HETE-PE by LC/MS and 15LO1 expression by Real-time PCR and Western-blot. (a) IL-13 induced 15LO1 mRNA levels rapidly return to basal levels at 24 hours after removal of IL-13. (b) high levels of 15LO1 protein remain for at least 72 hours following removal of IL-13. (c) intracellular 15-HETE-PE levels remain elevated over baseline after IL-13 removal.

553 Figure 3. 15LO1 knockdown suppresses IL-13 induced FOXA3 and MUC5AC while increasing
554 FOXA2 expression. HAECs with/without 15LO1 siRNA (siALOX15) transfections were
555 stimulated with IL-13 for 7 days, and cell lysates collected for protein and mRNA analysis. (a)
556 15LO1 knockdown suppressed IL-13 induced FOXA3 mRNA and (b) protein expression
557 (representative Western-blot and densitometry, n=4). (c) 15LO1 knockdown suppressed
558 MUC5AC mRNA and (d) protein expression induced by IL-13 (measured by ELISA, shown as
559 fold change related to scramble control). (e) 15LO1 knockdown upregulated FOXA2 mRNA.

560 Figure 4. BLX2477 treatment inhibited IL-13 induced FOXA3 and MUC5AC while increasing
561 FOXA2 expression. HAECs stimulated with IL-13 for 7 days were treated with BLX2477 at 2 μ M
562 for 5 days with DMSO as the vehicle control, and cell lysates collected for mRNA and protein
563 analysis. (a) BLX2477 decreased FOXA3 mRNA and (b) protein expression induced by IL-13.
564 (c) BLX2477 suppressed MUC5AC mRNA and (d) protein expression (measured by ELISA,
565 shown as fold change related to DMSO control) induced by IL-13. (e) BLX2477 increased
566 FOXA2 mRNA in response to IL-13 stimulation.

567 Figure 5. Suppression of 15LO1 levels and activity decreased IL-13 induced periostin
568 expression. HAECs stimulated with IL-13 were transfected with DsiRNA or treated with
569 BLX2477, and cell lysates collected for mRNA and protein analysis. 15LO1 knockdown (a, b)
570 and BLX2477 treatment (c, d) both suppressed IL-13 induced perisotin mRNA and protein
571 expression.

572 Figure 6. Exogenous 15-HETE-PE stimulation induced FOXA3, MUC5AC and periostin
573 expression in HAECs in the absence of IL-13. HAECs under ALI culture were stimulated with 1
574 μ M 15HETE-PE for 5 days, with DMPE applied as vehicle control. 15-HETE-PE induced FOXA3

575 (a, b), MUC5AC (c, d) and periostin (b, e), while no effect on FOXA2 (f). *Data presented as means*
576 *± SEM, analyzed by paired T-test.*

577

For Review Only

Figure 1

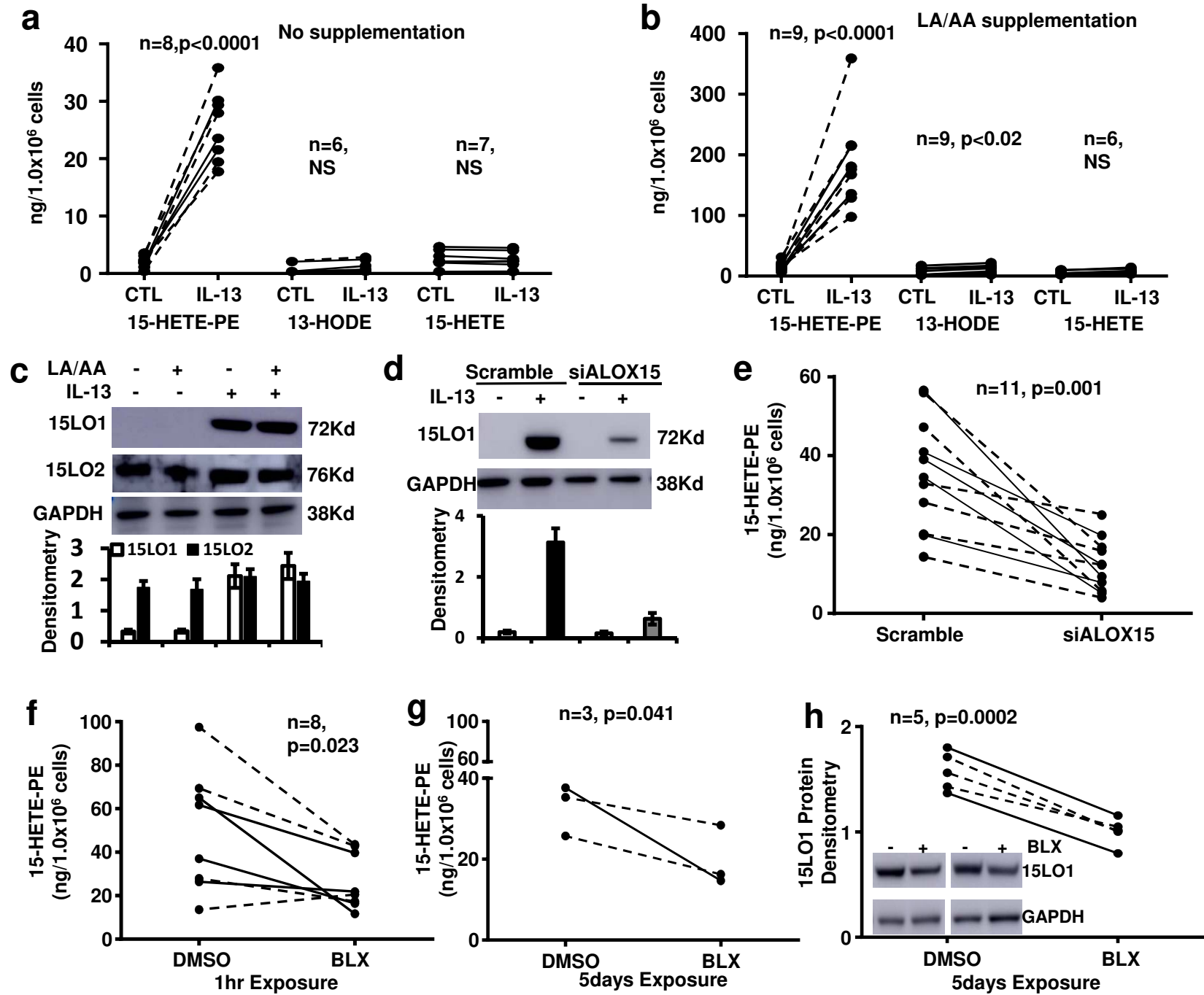


Figure 2

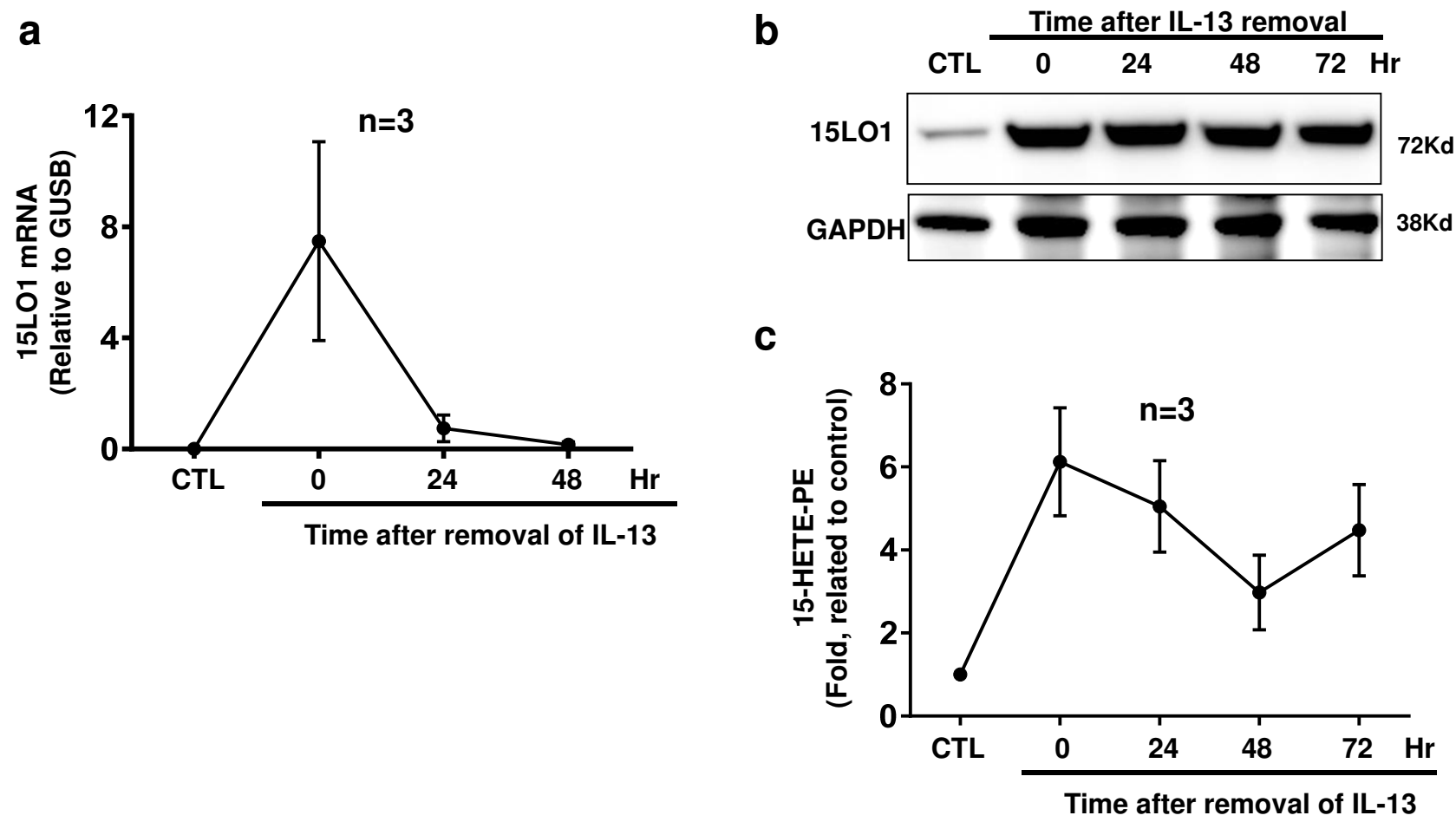


Figure 3

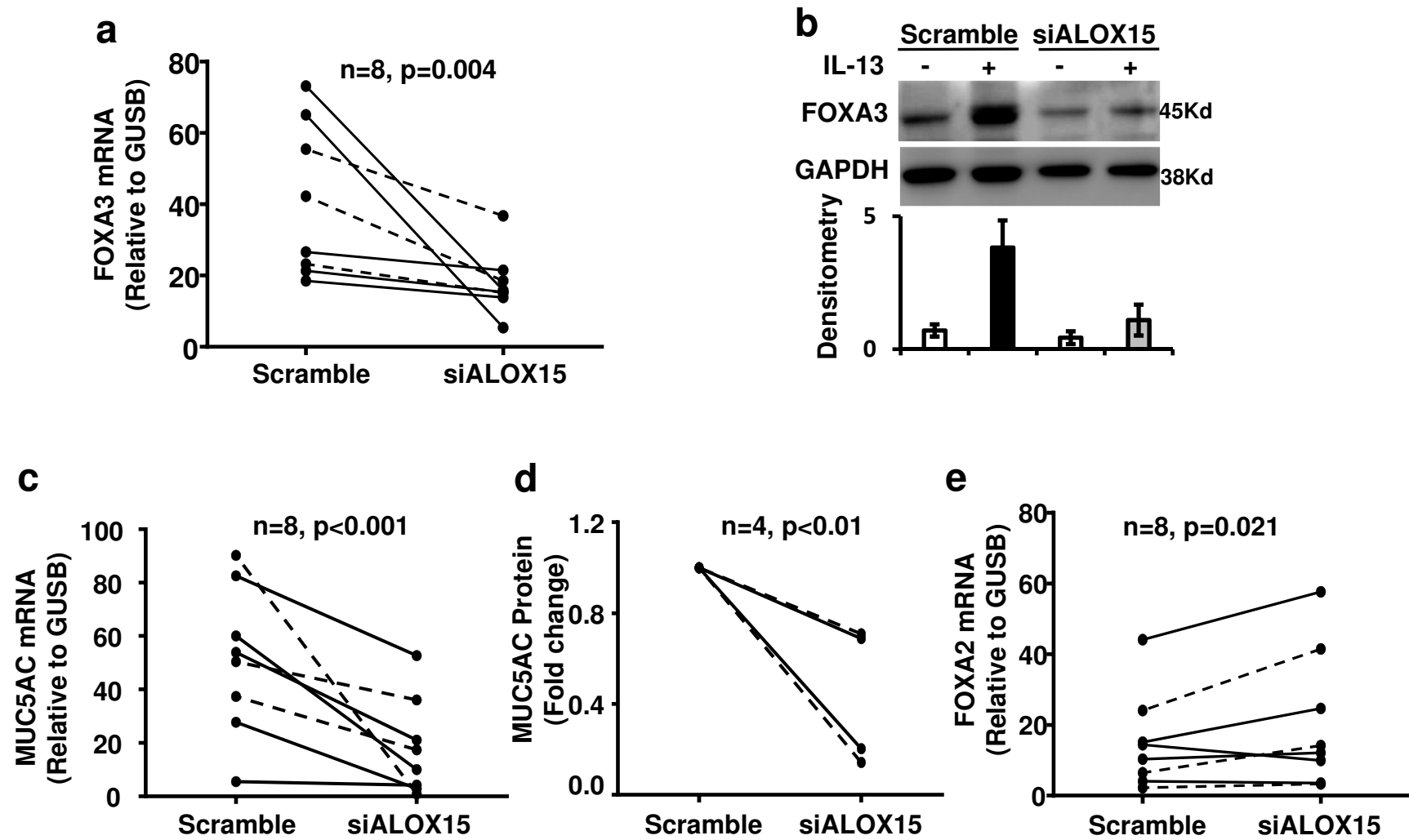


Figure 4

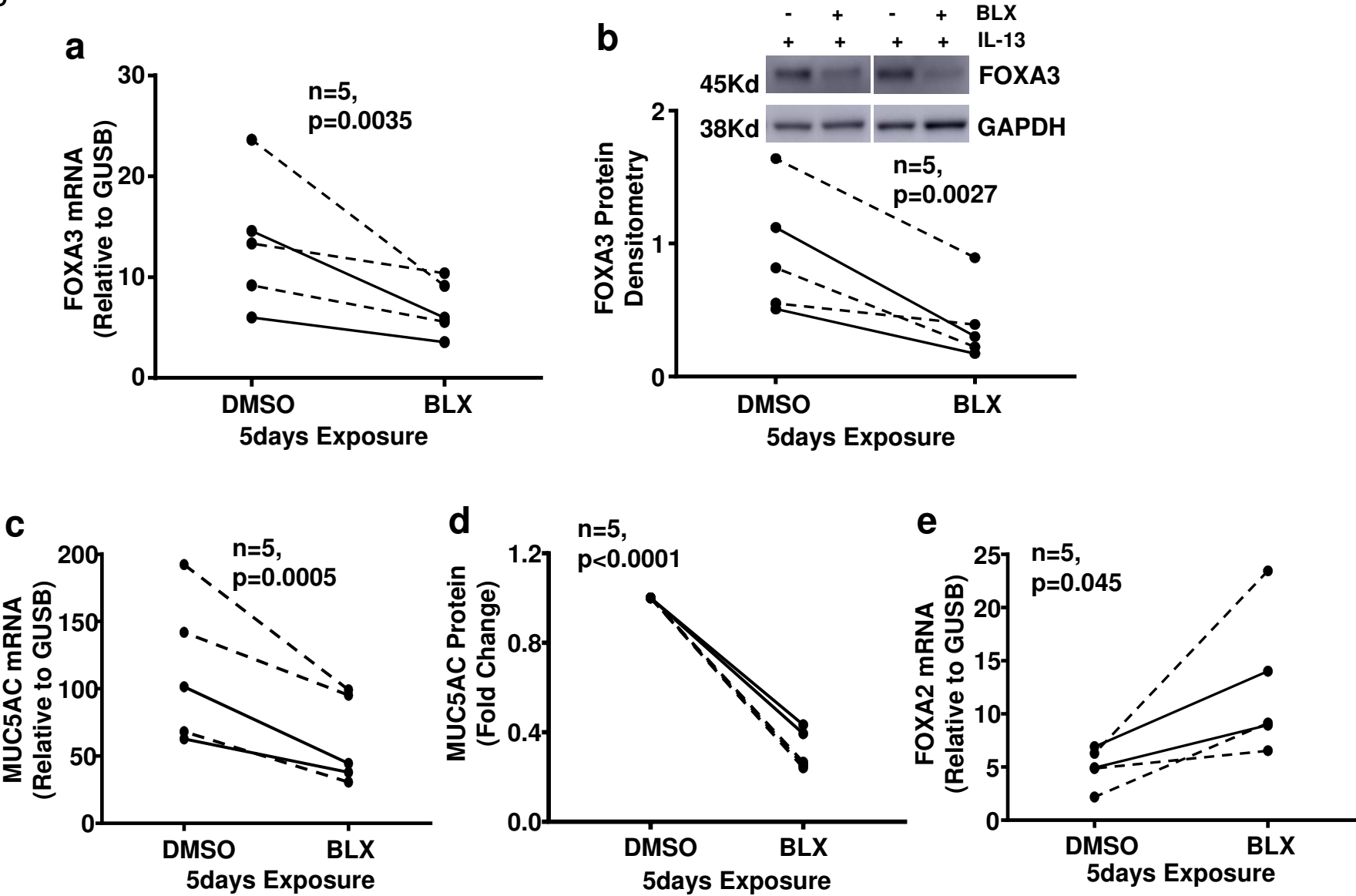


Figure 5

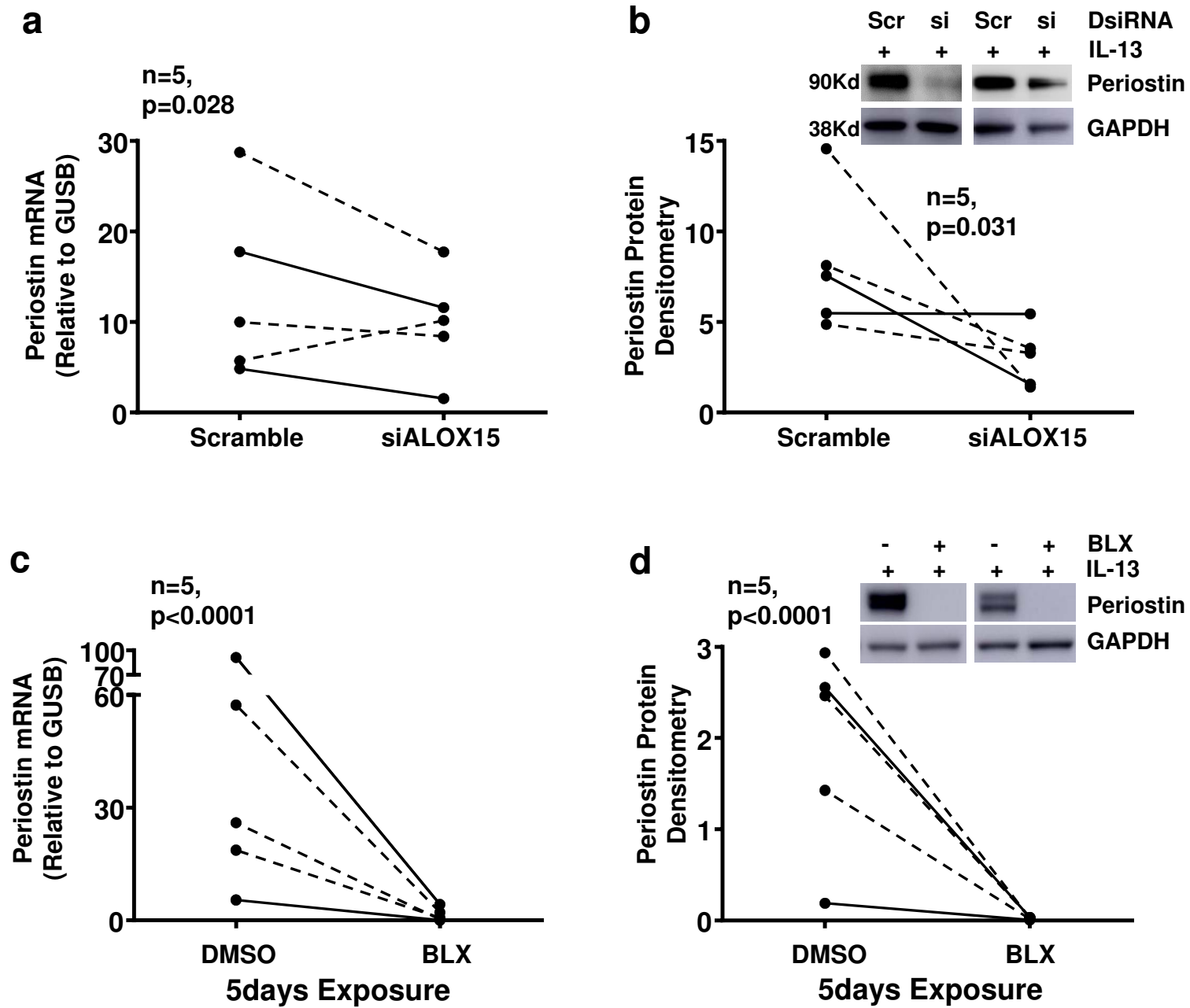
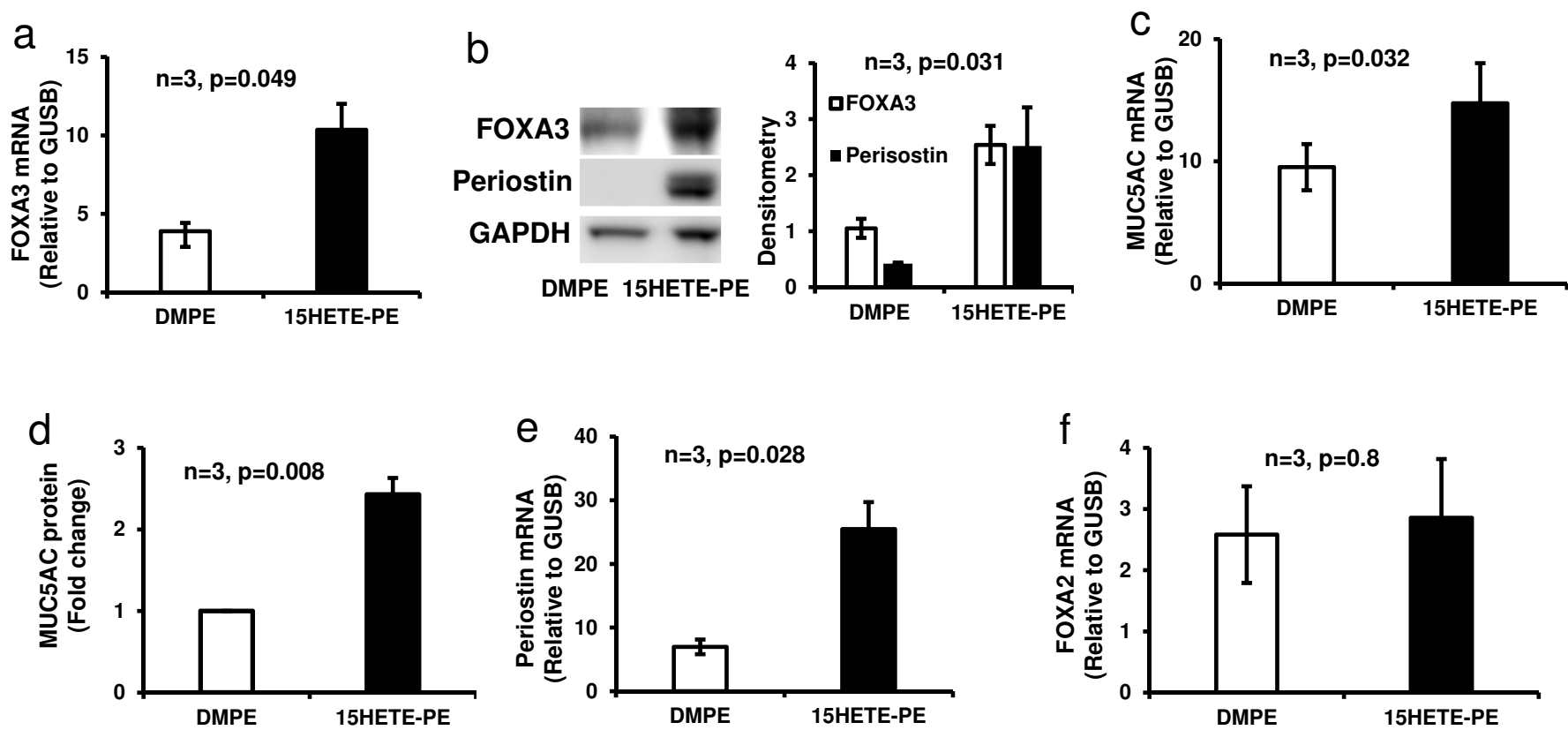


Figure 6



1 **Preferential generation of 15-HETE-PE induced by IL-13 regulates goblet cell**
2 **differentiation in human airway epithelial cells**

3 **Online Supplement**

4 **Materials and methods**

5 ***Reagents, antibodies and primers***

6 ALOX15 DsiRNATM was purchased from IDT (Coralville, IA), and *Lepofectamine* transfection
7 reagent was from Thermo Fisher (Rodkford, IL). Antibodies against FOXA3 (goat IgG) and
8 periostin (rabbit IgG1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-
9 GAPDH antibody was from Novus Biologicals (Littleton, CO). Anti-MUC5AC antibody was from
10 Neomarkers (Fremont, CA). Basic Epithelial Growth Medium (BEGM) cell culture medium and
11 supplements are purchased from Lonza (Basel, Switzerland). Recombinant human IL-13 was
12 purchased from R&D Systems (Minneapolis, MN). AA and LA were from Cayman Inc (Ann Arbor,
13 Michigan). 15LO1 antibody was a gift from Dr. Doug Conrad, University of California, San Diego(1).
14 15LO2 antibody was purchased from Abcam (Cambridge, MA). Real-Time PCR primers and
15 probes were all purchased from Applied Biosystems (Foster City, CA). BLX2477, a highly specific
16 inhibitor of 15LO1, was a kind gift from Dr. Hans-Erik Claesson (2).

17 ***Sources of HAECs***

18 HAECs were obtained by bronchoscopic brushing of asthmatic and healthy control (HC) airways
19 as previously described (3). All participants were recruited as part of the National Heart, Lung, and
20 Blood Institute's Severe Asthma Research Program or the Electrophilic Fatty Acid Derivatives in
21 Asthma studies (4). All asthmatic participants met American Thoracic Society (ATS) criteria for

asthma and included mild to severe asthmatic patients, while HCs were without respiratory disease and had normal lung function (5, 6). No subject smoked within the last year or >5 pack years. The study was approved by the University of Pittsburgh Institutional Review Board and all participants gave informed consent.

Primary Human Airway Epithelial Cell (HAEC) Culture in Air–Liquid Interface, DsiRNA Transfection and exogenous 15-HETE-PE stimulation

HAECs were cultured in air–liquid interface (ALI) under serum-free condition as previously described (5, 7). Briefly, fresh bronchoscopic brushing primary HAECs were cultured under immersed condition for proliferation. When 80-90% confluent, cells are trypsinized and plated at 5×10^4 cells per well on 12-well Transwell plate for submerged stage culture by adding 200 μ l culture medium to upper insert and 1000 μ l culture medium to lower chamber [BEBM/DMEM at 50:50, supplemented with 4 g/ml Insulin, 5 pg/ml Transferrin, 0.5 μ g/ml Hydrocortisone, 0.5 μ g/ml Epinephrine, 52 μ g/ml Bovine hypothalamus extract, 50 μ g/ml Gentamicin, 50 ng/ml Amphotericin, 0.5 μ g/ml albumine bovine, 80nM ethanolamine, 0.3 mM $MgCl_2$, 0.4 mM $MgSO_4$, 1 mM $CaCl_2$, 30 ng/ml retinoic acid and 0.5 ng/ml Epithelial Growth Factor (EGF)]. When cells reached 100% confluence, cells went into ALI culture for by reducing the upper volume to 50 μ l with the lower volume remaining at 1.0 mL full medium.

DsiRNA transfection was performed using Lipofectamine transfection reagent. Briefly, 50 nM DsiRNA was pre-mixed with 3 μ l/well Lipofectamine transfection reagent for 20 minutes at room temperature before pooled together with HAECs suspension and seeded onto transwells for incubations. After 24 hours, the transfection mixture was removed and cells were switched to ALI culture for 7 days. Cells were stimulated with IL-13 (10 ng/ml) under ALI culture for 7 days unless specified otherwise. For LA/AA supplementation, equal amounts of exogenous 10 μ M LA and 10

μ M AA were added into the basal culture medium for three days before harvest. For exogenous 15-HETE-PE stimulation, 1 μ M 15-HETE-PE [HPLC/MS collection dissolved in methanol as previously described (10)] was added into medium for culture for 5 days, with DMPE (Dimyristoyl-phosphoethanolamine) dissolved in methanol applied as vehicle control.

Semiquantitative MUC5AC ELISA

MUC5AC protein was measured from apical culture supernatants using a semiquantitative sandwich ELISA with two different MUC5AC antibodies, one for coating and one for detection. Briefly, high binding plates were coated with Neomarkers (Fremont, CA) MUC5AC antibody (1-13M1) at 1 μ g/ml. Neomarker MUC5AC antibody (45M1) labeled with biotin was used at 0.2-0.4 μ g/ml for detection. The MUC5AC standard was generated from the apical supernatants of IL-13 stimulated HAECs cells under ALI and diluted 1/100 for the high standard followed by serial half-dilution. Thus, all results are in relative arbitrary units per ml (AU/ml) and semi-quantitative. Samples were studied without dilution or up to 1/4000 dilution depending on sample/system. Thus, the MUC5AC results were reported as fold change in relative to corresponding control condition.

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86 phosphatidylethanolamine-binding protein to regulate MAPK signaling in human airway epithelial cells.
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97 Supplement Figure Legends

98 Figure S1. IL-13 stimulation induces modest increases in free 15-HETE and 13-HODE in cell
99 culture media in the presence of equal LA/AA supplement in HAECs. HAECs were stimulated
100 with IL-13 for 7 days with LA/AA supplements, and culture media collected for 13-HODE and 15-
101 HETE measurement by LC/MS.

102 Figure S2. IL-13 induced FOXA3 expression while downregulating FOXA2 mRNA expression.
103 HAECs were stimulated with IL-13 for 3 to 7 days, and cell lysates collected for protein and
104 mRNA analysis. IL-13 induced FOXA3 expression which paralleled the increase of 15LO1 (a, b),
105 while suppressed FOXA2 expression (c).

106 Figure S3. IL-13 induced periostin (a) mRNA, (b) protein expression and (c) secretion. HAECs
107 were stimulated with IL-13 for 7 days, and cell lysates and lower chamber culture media
108 collected for mRNA and protein detection. *For Western-blot in Figure S3c, 50 μ l of culture media*
109 *was loaded in each sample.*

110 Figure S4. 15LO1 suppression decreases periostin secretion induced by IL-13 in HAECs. HAECs
111 with IL-13 stimulation were transfected with DsiRNA or treated with BLX2477, and culture media
112 collected for protein detection. 15LO1 knockdown (a) and BLX2447 treatment (b) suppressed
113 perisotin secretion induced by IL-13. *50 μ l of culture media was loaded in each sample.*

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Table S1

Demographics of research participants providing HAECs for culture

| | Healthy n=18 | Mild/Moderate n=16 | Severe n=10 |
|--|-----------------|-----------------------|----------------|
| Gender (M/F) | 5/13 | 5/11 | 4/6 |
| Age (year), median ± SE | 30.5 ± 2.9 | 29 ± 3.6 | 49 ± 1.6 |
| Race (C/AA/O) | 17/0/1 | 6/10/0 | 7/2/1 |
| FeNO (ppb), median ± SE | 12 ± 1.7 | 17 ± 6.1 | 41 ± 9.6 |
| FEV ₁ (%predicted), median ± SE | 102 ± 1.5 | 92 ± 3.5 | 79 ± 5.8 |
| ICS (yes/no) | NA | 11/5 | 10/0 |

Definition of abbreviations: FeNO = Fractional exhaled nitric oxide;
ICS = Inhaled corticosteroid; M/F = Male/Female; C/AA/O = Caucasian/African American/Others;
NA = Not applicable

Figure S1

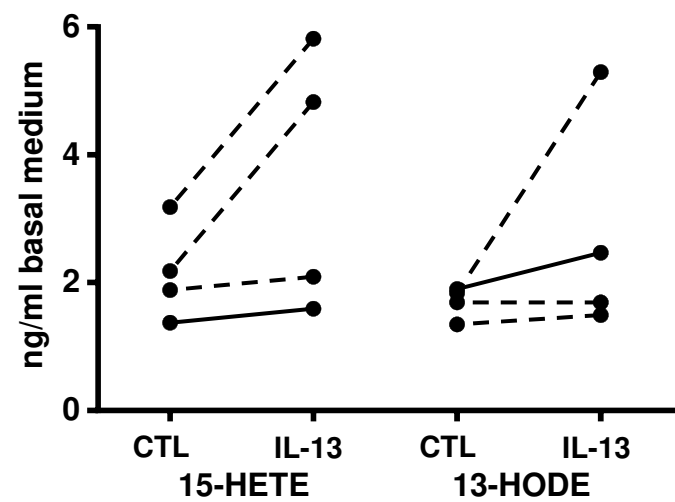


Figure S2

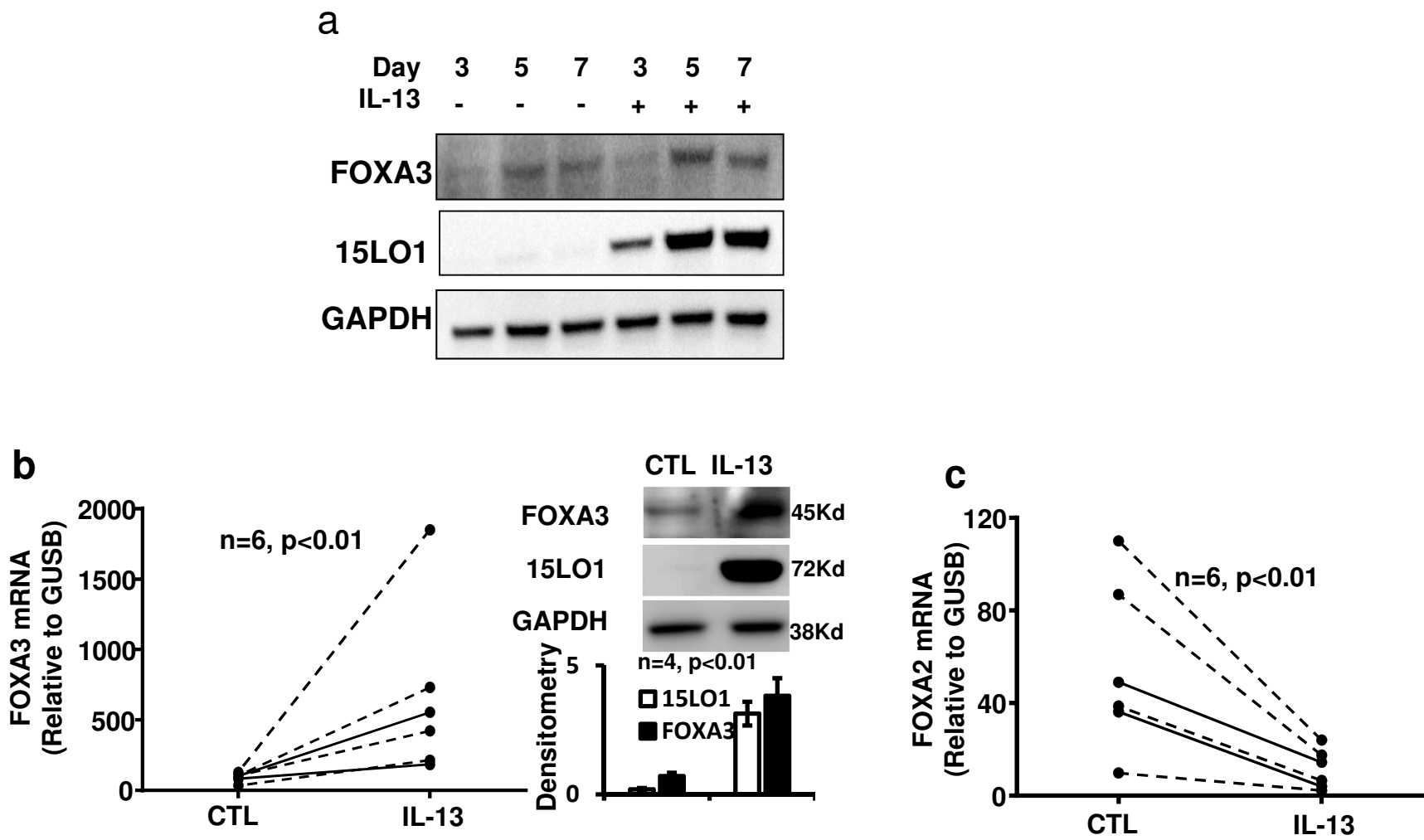


Figure S3

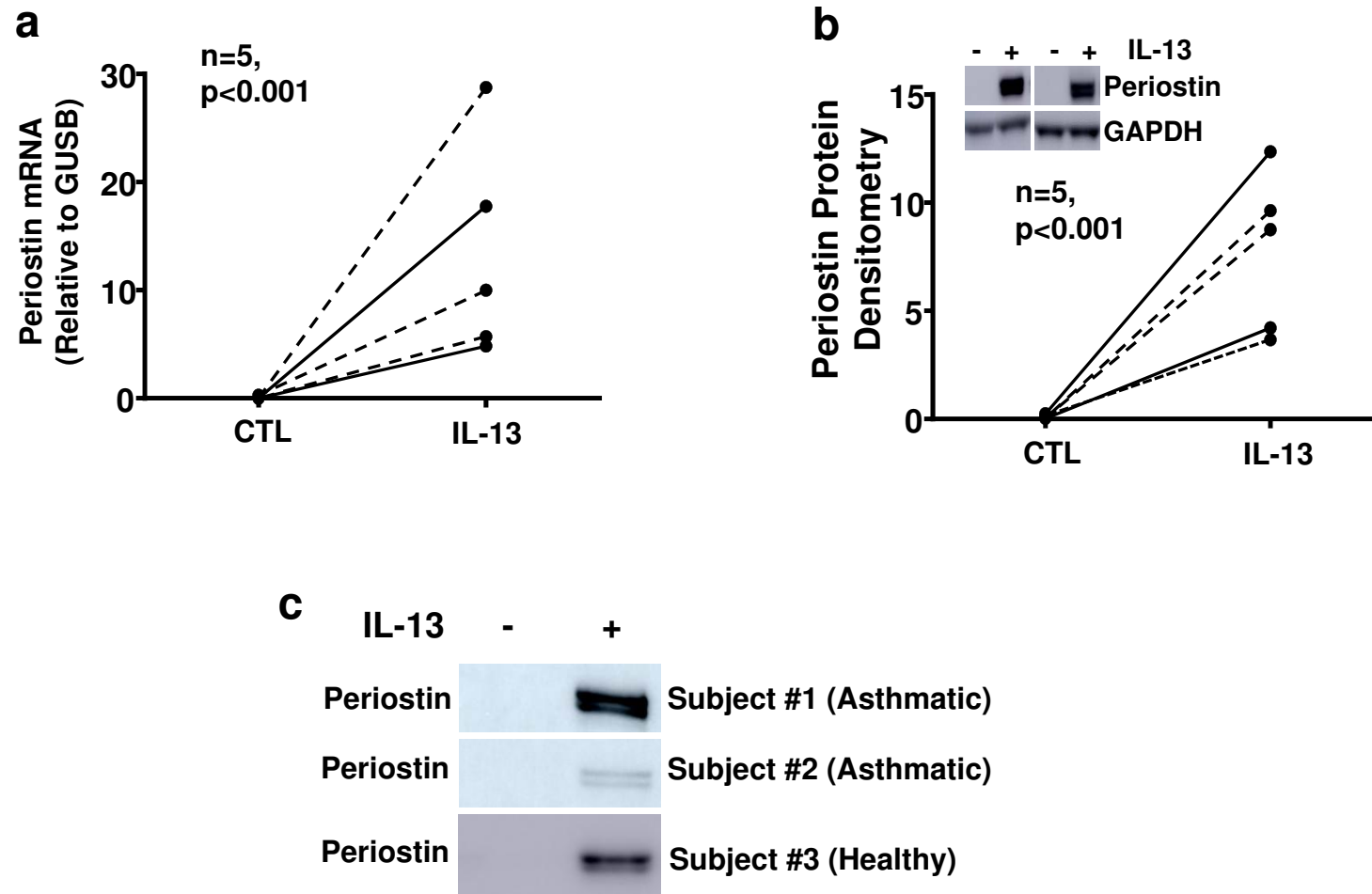


Figure S4

